SHORT COMMUNICATION

The Cellular Location of Proteases in *Candida albicans*

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Vacuoles prepared from yeast cells of *Candida albicans* were enriched in proteinase ycaB (EC 3.4.21.48) but not in aminopeptidase or β -glucosidase. Proteinase ycaB, assayed *in situ*, increased 1.5-fold during starvation whereas aminopeptidase activity decreased by 25%. Proteinase ycaB increased a further 1.5-fold during germ-tube formation.

INTRODUCTION

In the dimorphic yeast *Candida albicans*, development of germ-tubes from yeast cells is the initial stage in the yeast-mycelium transition. *De novo* protein synthesis during germ-tube formation has been studied but the function of the yeast's proteolytic system has not been considered. In this paper, we describe the cellular location of proteinase ycaB (Farley *et al.*, 1986) and an aminopeptidase (Montes & Constantine, 1968) together with an analysis of the levels of these enzymes during morphogenesis.

METHODS

Yeast strains and growth media. *Candida albicans* (ATCC 10261) was grown as described previously (Farley *et al.*, 1986) and germ-tube formation induced with starved yeast cells and *N*-acetylglucosamine at 37 °C (Ram *et al.*, 1984). Starved cells incubated at 28 °C under these conditions retained the yeast morphology and are referred to as yeast control cells. *Saccharomyces cerevisiae* strains X2180-1A (Yeast Genetics Stock Center, Berkeley, CA) and ABYS1 (kindly provided by D. H. Wolf, Freiburg, FRG) were grown on YPD medium (Emter & Wolf, 1984).

Preparation of vacuoles. Concanavalin-A-stabilized spheroplasts were prepared as described by Hubbard *et al.* (1986) except that 0.25×10^{10} rather than 10^{10} cells were used. Metabolic lysis was performed as described by Braun & Calderone (1978) except that the buffer was 0.1 M-Tris/HCl, pH 7.2, containing 0.3 M-mannitol. After centrifugation (500 g, 10 min) to remove unlysed cells, the supernatant was centrifuged (60 000 g, 90 min) to obtain a mixed membrane fraction which was resuspended in 0.1 M-Tris/HCl buffer, pH 7.2, containing 40% (w/v) sucrose (10 ml for a preparation commencing with 0.25×10^{10} cells). This was layered onto 3.5 ml 50% (w/v) sucrose, overlaid with buffered sucrose solutions (8 ml each of 35%, 25% and 10%) and centrifuged (60 000 g, 90 min) at 4 °C in a swing-bucket rotor (Beckman SW27). The material in the top 1.0 ml of the gradient was collected and stored on ice for subsequent analysis.

Enzyme assays. Proteinase ycaB (EC 3.4.21.48) was assayed with Cbz-Leu-ONp (100 μ M) as substrate (Farley *et al.*, 1986) except that the buffer was 0.1 M-Tris/HCl, pH 7.2, containing 10% (w/v) sucrose. For the *in situ* assay the buffer was 100 mM-sodium acetate, pH 6.0, containing 1 mM-EDTA and the total assay volume 10.1 ml. Immediately after the addition of cells (usually 0.13 – 0.25×10^9) and again at 5–10 min intervals, 2 ml samples were cooled on ice, centrifuged (2500 g, 7 min) to remove the cells and the absorbance of each supernatant was measured at 348 nm. α -D-Mannosidase (EC 3.2.1.24) was assayed with Meu- α -D-mannoside (0.4 mM) as substrate in 40 mM-KH₂PO₄/NaOH buffer, pH 6.5 (total volume 0.25 ml). After 30 min at 37 °C the reaction was stopped

Abbreviations: Cbz-, benzyloxycarbonyl-; Meu-, 4-methylumbelliferyl-; -ONp, *p*-nitrophenyl ester; -Nna, β -naphthylamide.

by the addition of 0.5 ml 0.1 M-glycine/NaOH buffer, pH 10.3 and the fluorescence of the 4-methylumbelliferone measured (excitation wavelength 382 nm; emission wavelength 450 nm). β -Glucosidase (EC 3.2.1.21) was assayed under the same conditions with Meu- β -D-glucoside. Acid phosphatase (EC 3.1.3.2), alkaline phosphatase (EC 3.1.3.1), isocitrate dehydrogenase (NADP⁺) (EC 1.1.1.42) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were assayed as described by Ram *et al.* (1983) except that for glucose-6-phosphate dehydrogenase each assay also contained 50 μ mol glucose 6-phosphate and 0.3 μ mol NADP⁺. Succinate dehydrogenase (EC 1.3.99.1) was assayed as described by Hubbard *et al.* (1986). NADPH:cytochrome *c* reductase (EC 1.6.2.4) was assayed as described by Masters *et al.* (1967). Carboxypeptidase activity in cell-free extracts prepared by mechanical disruption of yeast cells in 0.1 M-KH₂PO₄/NaOH buffer, pH 7.0, containing 0.1% sodium azide was assayed (30 °C) with Cbz-Phe-Leu (Hayashi, 1976). Aminopeptidase activity was assayed with Leu-Nna as substrate (109 μ M) in 0.1 M-imidazole/HCl buffer, pH 7.0 (total volume 0.25 ml). After 60 min at 37 °C the reaction was stopped (100 °C, 5 min), the mixture was diluted with 0.1 M-Tris/HCl buffer, pH 7.2, containing 10-mM MgCl₂ (0.5 ml) and the fluorescence of the β -naphthylamine measured (Farley *et al.*, 1986).

Detection of extracellular proteinase activity. Cultures of exponentially growing, starved or germ-tube-forming cells were centrifuged (3000 g, 10 min). The supernatant was filtered (1.2 μ m), adjusted to pH 6.0–6.5, concentrated by dialysis against flaked polyethylene glycol (overnight, 4 °C) and tested for proteinase activity (24 h, 37 °C) with denatured [³⁵S]protein as substrate (Farley *et al.*, 1986).

RESULTS AND DISCUSSION

Cellular location of enzymes

No proteinase ycaB was detected in concentrated culture filtrates from either yeast cells or germ-tube-forming cells. Pepstatin-sensitive proteinase activity in these preparations probably represents proteinase ycaA (Remold *et al.*, 1968). Vacuole preparations were enriched in α -D-mannosidase (marker enzyme for the vacuolar membrane; Schekman, 1982), alkaline phosphatase (marker enzyme for the soluble contents of the yeast vacuole) and proteinase (Table 1). The vacuole fraction was not significantly contaminated by other cell organelles. Enzyme markers for the cytoplasm (glucose-6-phosphate dehydrogenase), the mitochondrial matrix (isocitrate dehydrogenase), the mitochondrial membranes (succinate dehydrogenase), secretory vesicles (acid phosphatase) and the endoplasmic reticulum and nuclear membrane (NADPH:cytochrome *c* reductase) were either not detectable or only present as minor contaminants. [³H]Concanavalin A, used to stabilize the spheroplasts before lysis, was absent from the vacuole fraction. These results are comparable with those reported by Wiemken *et al.* (1979) for crude vacuoles from *S. cerevisiae* and establish that a proteinase which hydrolyses Cbz-Leu-ONp at neutral pH (proteinase ycaB) is present in the vacuoles from yeast cells of *C. albicans*. The pH of the vacuole is around 6.0 (Cassone *et al.*, 1983) so that the proteinase should be active *in vivo*.

In *S. cerevisiae* not only proteinase yscB but also proteinase yscA, carboxypeptidases Y and S and two aminopeptidases are located in the vacuole of the yeast cell (Emter & Wolf, 1984). No carboxypeptidase activity was detected in either the lysate or the vacuolar fraction but it is most likely that *C. albicans*, like *S. cerevisiae*, contains a vacuolar carboxypeptidase and a cytoplasmic inhibitor of this enzyme: carboxypeptidase activity was detected in cell-free extracts (0.07 μ mol min⁻¹ mg⁻¹) and the specific activity increased 47-fold during a 19 h incubation at pH 5.0 and 30 °C. The carboxypeptidase was inhibited by phenylmethylsulphonyl fluoride (94% at 5 mM). Montes & Constantine (1968) found Leu-Nna-hydrolysing activity in the vacuoles, in the cytoplasm and on the external surface of the cell wall. In the present study no aminopeptidase activity was detectable in the vacuole preparation. All the activity (103%) in the lysate was recovered in the 60000 g supernatant (specific activity 20 pmol min⁻¹ μ g⁻¹). Atmanspacher & Rohm (1981) reported that *Schizosaccharomyces pombe* does not contain a vacuolar aminopeptidase. A similar result (105% recovery in the 60000 g supernatant, specific activity 13 pmol min⁻¹ μ g⁻¹) was obtained for β -glucosidase in *C. albicans*. Since β -glucosidase is not periplasmic (Ram *et al.*, 1984) it must be a cytoplasmic enzyme. The aminopeptidase activity in the 60000 g supernatant exhibited optimum activity for Leu-Nna between pH 7.0 and 8.0, which is similar to the pH optima for the periplasmic and cytoplasmic aminopeptidases in *S. cerevisiae* (Frey & Rohm, 1979). The rates of hydrolysis (nmol min⁻¹ ml⁻¹) of a series of β -naphthylamide substrates (140 μ M) were: Leu-Nna, 15; Pro-Nna, 13; Arg-Nna, 13; Ala-Nna, 11; Phe-Nna, 9; Leu-4-methoxy-Nna, 6; Met-Nna, 5.

Table 1. *Enzyme activities in the spheroplast lysate and the vacuole fraction of C. albicans*

The lysate (500 g supernatant) and vacuole fraction were prepared and enzyme activities measured at least in duplicate as described in Methods. For the measurement of radioactivity, samples (up to 0.1 ml) were added to 0.6 ml NCS tissue solubilizer (Amersham) and incubated at 37 °C for 20 min with shaking prior to addition of 10 ml scintillant (6 g 2,5-diphenyloxazole per litre of toluene). Protein was measured using the dye-binding assay (method 1) described by Bearden (1978).

Enzyme	Specific activity (pmol min ⁻¹ µg ⁻¹)	
	Lysate	Vacuole
α-Mannosidase	10	60
Alkaline phosphatase	125	1600
Proteinase ycaB	80	1200
Glucose-6-phosphate dehydrogenase	880	0
Isocitrate dehydrogenase	26	0
Succinate dehydrogenase	55	4
NADP:cytochrome c reductase	40	13
Acid phosphatase	25	15
β-Glucosidase	8	<0.3*
Aminopeptidase	13	<4*
[³ H]Concanavalin A	65†	0†

* Estimated detection limits. † Radioactivity (c.p.m. µg⁻¹).

Table 2. *Proteinase ycaB and aminopeptidase activity in different cell types*

Cells were prepared as described in Methods, harvested by centrifugation (2500 g, 10 min) and washed twice with water. Proteinase ycaB was measured *in situ* as described in Methods using intact cells washed twice with assay buffer. The results are means of a least duplicate determinations for two separate experiments. Aminopeptidase activity was measured in cell-free extracts prepared in 0.1 M-Tris/HCl buffer, pH 7.2, containing 10 mM-MgCl₂, 10% (w/v) sucrose and 0.02% sodium azide using a Braun homogenizer (Farley *et al.*, 1986). The homogenate was centrifuged at 1000 g for 10 min and again at 60 000 g for 90 min. The 60 000 g supernatant was assayed for enzyme activity and the protein concentration measured by an alternate modified Lowry method (Albro, 1975). The results are means of duplicate determinations for a single experiment. Units of enzyme activity are nmol min⁻¹.

Cell type	Proteinase ycaB [units (10 ⁹ cells) ⁻¹]	Aminopeptidase	
		[units (10 ⁹ cells) ⁻¹]	[units (mg protein) ⁻¹]
Exponential-phase yeast cells	56 ± 7%	40	10
Starved yeast cells	81 ± 9%	27	8
Germ-tube-forming cells	126 ± 14% (3h)	34 (1 h) 43 (4 h)	9 (1 h) 11 (4 h)
Yeast control cells	84 ± 14% (3 h)	ND	ND

ND, Not determined.

Enzyme activity during morphogenesis

Since no endogenous inhibitor of the aminopeptidase was detected, enzyme activity in different cell types was investigated using cell-free extracts. The aminopeptidase activity (Table 2) decreased 25% during starvation but returned to the original level during germ-tube formation. This may reflect decreased synthesis and/or increased degradation of the enzyme under these conditions. Proteinase ycaB cannot be measured in cell-free extracts until the endogenous inhibitor is destroyed (Farley *et al.*, 1986); however, enzyme activity can be measured *in situ* with intact cells and Cbz-Leu-ONp as substrate (Table 2) as judged by the following observations. Enzyme activity measured *in situ* in *S. cerevisiae* ABYS1 (a strain which lacks proteinases yscB and yscA) was 11% of that measured in the wild-type strain X2180-1A [26 nmol min⁻¹ (10⁹ cells)⁻¹]. Acid treatment (Ram *et al.*, 1984) of *C. albicans* cells did not

inactivate the *in situ* proteinase activity but a crude proteinase ycaB preparation was inactivated at pH 1.5 and 0 °C. The *in situ* proteinase activity was not increased by permeabilizing (Ram *et al.*, 1984) cells. Intact cells are also permeable to Leu-Nna (Montes & Constantine, 1968). The *in situ* proteinase activity was inhibited by preincubation (4 °C, 10 h) of the cells with phenylmethylsulphonyl fluoride (2 mM) or mercuric chloride (1.2 mM), both typical inhibitors of proteinase ycaB.

Proteinase ycaB activity increased 1.5-fold during starvation and a further 1.5-fold during germ-tube formation, whereas the amount of enzyme in yeast control cells remained unchanged (Table 2). It has been suggested that proteinase ycaB is involved in general protein degradation (Farley *et al.*, 1986), in which case the elevated levels of this enzyme during germ-tube formation suggest that general protein turnover is important during this morphological change and that protein degradation rates during germ-tube formation should be investigated.

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